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(54) Title: METHODS FOR ENHANCING THE PRODUCTION OF CYTOKINES IN CELL CULTUR E

(57) Abstract: The present invention is directed to methods for enhancing the production of cytokines in mammalian cell culture by modifying the level of a cytokine regulatory factor in the cells, resulting in enhanced levels of cytokine production. The invention is further directed to cell lines which exhibit enhanced cytokine regulatory factor expression and enhanced cytokine expression.

METHODS FOR ENHANCING THE PRODUCTION OF CYTOKINES IN CELL CULTURE

Field Of The Invention

The present invention relates to methods for enhancing the production of cytokines in cell culture by the over-expression of PKR.

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Background Of The Invention

dsRNA-activated protein kinase (PKR) referred to as P1/eIF2 kinase, DAI or dsI for dsRNA-activated inhibitor, and p68 (human) or p65 (murine) kinase, is a serine/threonine kinase whose enzymatic activation requires binding to dsRNA or to single-stranded RNA presenting internal dsRNA structures and consequent autophosphorylation (Galabru and Hovanessian, 1987; Meurs, *et al.*, 1990). Analogous enzymes have been described in rabbit reticulocytes, different murine tissues, and human peripheral blood mononuclear cells (Farrel, *et al.*, 1977; Levin, *et al.*, 1978; Hovanessian, 1988; Krust, *et al.*, 1982; Buffet-Janvresse, *et al.*, 1986).

PKR has been shown to play a variety of important roles in the regulation of translation, transcription, and signal transduction pathways through its ability to phosphorylate protein synthesis initiation factor eIF2, and I-kappaB (the inhibitor of NF-kappaB; Kumar A, *et al.*, 1994), in addition to other substrates.

The best characterized *in vivo* substrate for PKR is the alpha subunit of eukaryotic initiation factor-2 (eIF-2alpha) which, once phosphorylated, ultimately leads to inhibition of cellular and viral protein synthesis (Hershey, 1991). PKR has been demonstrated to phosphorylate initiation factor eIF-2 alpha *in vitro* when activated by double-stranded RNA (Chong, *et al.*, 1992).

Activities attributed to PKR include a role in (1) mediating the antiviral and anti-proliferative activities of IFN-alpha and IFN-beta, (2) the response of uninfected cells to physiologic stress, and (3) cell growth regulation (Clemens MJ and Elia A, 1997; Zamanian-Daryoush M, *et al.*, 1999).

It has also been suggested that PKR may function as a tumor suppressor and inducer of apoptosis. (See, *e.g.*, Clemens MJ and Bommer UA, 1999; Yeung, Lau et al, 1996; Koromilas *et al.*, 1992), with recent results indicating that expression of an active form of PKR triggers apoptosis, possibly through upregulation of the Fas receptor (Donze O, *et al.*, 1999).

It has further been shown that when a cell line capable of producing interferon is transfected with an expression vector encoding PKR, over-expression of PKR induces increased production of interferon (WO 97/08324). The interferon family of proteins are prototypes of cytokines and have been shown to play key roles in immune defense against pathogens and cancerous tissues.

Cytokines are regulatory molecules which exhibit a range of biological activities and act on a wide range target cells. (See, *e.g.*, Balkwill FR and Burke F, 1989; Wong G and Clark S, 1988; and Clark S and Kamen R, 1987.)

In general, cytokines are currently produced by the expression of recombinant proteins in insect, bacterial and mammalian host cells.

Cytokines are produced for various therapeutic applications by either purifying the natural cytokine from mammalian cell lines or recombinantly producing the cytokine in insect, microbial or mammalian cells. Natural cytokines are preferable in that they are known to contain the full repertoire of native forms of a given cytokine and have the proper structure, but they are expensive and time-consuming to produce.

Recombinantly produced cytokines are less expensive to make, but dependent upon the source may contain foreign antigens, resulting in an immune response by the subject to which they are administered, or may be less active due to structural variation from the native form, *i.e.*, glycosylation pattern.

Thus, a method for enhancing the production of natural cytokines to make them less expensive to produce would be advantageous.

Present methods utilize expression of cytokines in microbial systems, which do not permit the glycosylation and native folding of the cytokine proteins, or in human cells that generally produce very low levels of recombinant protein.

The present invention is directed to the surprising discovery that by manipulating the expression of certain genes, cytokine production is enhanced in cultured mammalian cells.

Summary Of The Invention

The invention provides methods for enhanced production of cytokines in mammalian cell culture by providing cell lines in which the expression of a cytokine regulatory protein is increased to above-normal levels, resulting in an increase in cytokine production.

In one aspect, the invention provides a method for producing a cytokine by culturing a mammalian cell line that has been modified in an manner to result in overexpression of a cytokine regulatory factor and treated to effect cytokine production, followed by collection of the cytokine produced by the cultured, treated cell line.

In one preferred embodiment, the cytokine regulatory factor is PKR.

In one approach, the mammalian cell line is transfected with an expression vector having a promoter fragment which functions in the host cell, operably linked to a DNA segment encoding a cytokine regulatory factor, such that the cytokine regulatory factor is overexpressed in the cell line.

The cell line may be treated by priming and inducing to effect cytokine production, *e.g.* by priming with a phorbol ester, such as phorbol myristate acetate (PMA), and by inducing with a ds-RNA such as poly r(I):poly r(C). Priming is a well known phenomenon whereby pretreatment of cells with a cytokine or other compound results in enhanced production of the same and/or additional cytokines following subsequent stimulation.

Exemplary cytokines the expression of which may be increased using the methods of the invention include, but are not limited to, interleukins (IL-1α IL-1β, IL-1ra, IL-2 and IL-4 through 8), tumor necrosis factors alpha and beta (TNF-β), the colony stimulating factors (granulocyte colony stimulating factor, G-CSF; granulocyte-macrophage colony stimulating factor, GM-CSF; and IL-3), the angiogenic factors (fibroblast growth factor, FGF; vascular endothelial growth factor, VEGF; and platelet-derived growth factors 1 and 2 (PDGF-1 and -2) and the anti-angiogenic factors (angiostatin and endostatin).

The cytokines may be expressed under the control of an inducible promoter, *e.g.*, a metallothionein promoter or a tetracycline (TRE) promoter; or a constitutive promoter, *e.g.*, a CMV promoter.

The invention further provides cell line compositions characterized by above normal expression of a cytokine regulatory factor, *e.g.*, PKR, and above normal expression of a cytokine, *e.g.*, interleukin 6 (IL-6), interleukin 8 (IL-8) or tumor necrosis factor beta (TNF-β).

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples.

Brief Description Of The Figures

Figure 1 illustrates interleukin-6 (IL-6) production in untransformed Namalwa and PKR-overexpressing Namalwa cells. 2A1.D1.G7 and parental Namalwa cells were cultured in DMEM/F12 medium containing 20 nM PMA for 20 hr followed by treatment with 200 μg/ml poly r(I):r(C) for 3 days (+), or left untreated (-). Culture supernatants were collected and analyzed for IL-6 production by ELISA (R&D Systems), according to the procedure provided by the supplier.

Figure 2 illustrates interleukin-8 (IL-8) production in untransformed Namalwa and PKR-overexpressing Namalwa cells, following the same procedure set forth in the description for Figure 1, except that the culture supernatants were analyzed for IL-8 production by ELISA according to the procedure provided by the supplier of the ELISA kits (R&D Systems).

Figure 3 illustrates tumor necrosis factor β (TNF-β) production in untransformed Namalwa and PKR-overexpressing Namalwa cells, following the same procedure set forth in the description for Figure 1, except that the culture supernatants were analyzed for TNF-β production by ELISA according to the procedure provided by the supplier of the ELISA kits (R&D Systems).

Detailed Description Of The Invention

1. Definitions

The term "vector" refers to a nucleotide sequence that can assimilate new nucleic acids, and propagate those new sequences in an appropriate host. Vectors include, but are not limited to recombinant plasmids and viruses. The vectors (*e.g.*, plasmid or recombinant virus) of the invention can be in a carrier, for example, a plasmid complexed to a protein, a plasmid complexed with lipid-based nucleic acid transduction systems, or other non-viral carrier systems.

An expression vector may comprise additional elements, for example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. Further, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and

preferably two homologous sequences that flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

Expression and cloning vectors typically contain a selectable marker gene, which encodes a protein that (a) confers resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complements auxotrophic deficiencies, or (c) supplies critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

Within the vector, a nucleic acid coding sequence must be "operably linked" by placing it in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" DNA sequences are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters combine elements of more than one promoter, are generally known in the art, and are useful in practicing the present invention.

As used herein, the term "cytokine regulatory factor expression" refers to transcription and translation of the cytokine regulatory factor gene, the products of which include precursor RNAs, mRNAs, polypeptides, post-translation processed polypeptides, and derivatives thereof, including cytokine regulatory factors from other non-human species such as murine or simian enzymes. As used herein, the term "PKR expression" refers to transcription and translation of a PKR gene, the products of which include precursor RNAs, mRNAs, polypeptides, post-translation processed polypeptides, and derivatives thereof, including PKRs from other non-human species such as murine or simian enzymes.

As used herein, the terms "biological activity of PKR" and "biologically active PKR" refer to any biological activity associated with PKR, or any fragment, derivative, or analog of PKR, such as enzymatic activity, specifically including autophosphorylation activity, eukaryotic translation initiation factor 2 (eIF-2) phosphorylation activity, or kinase activity

resulting in enhanced transcription of protein such as cytokines. It follows that the biological activity of a given cytokine regulatory factor refers to any biological activity typically attributed to that factor by those of skill in the art.

As used herein, the terms "normal level of cytokine regulatory factor activity", "normal level of cytokine regulatory factor expression", exemplified by "normal level of PKR activity" and "normal level of PKR expression" refer to the level of cytokine regulatory factor, *e.g.*, PKR activity or expression, determined to be present in unstimulated or uninfected cells of a particular type, *e.g.*, a particular cell line. It will be appreciated that such "normal" cytokine regulatory factor activity or expression, is reported as a range of cytokine regulatory factor activity or expression which is generally observed for a given type of cells which are not transfected with a vector encoding the cytokine regulatory factor, unstimulated (not induced or primed) and uninfected and may vary somewhat dependent upon culture conditions.

For example, the U937 cell line may have a normal range of PKR activity which differs from the normal range of PKR activity for the U937, T98G or Namalwa cell lines. It follows that over-expression of a given cytokine regulatory factor, *e.g.*, PKR, means an expression level which is above the normal range of PKR expression generally observed for a given type of cells which are not transfected with a vector encoding PKR, unstimulated (not induced, pretreated, or primed) and uninfected.

Similarly, as used herein, the terms "normal level of cytokine activity" and "normal level of cytokine expression" refer to the level of cytokine activity or expression, determined to be present in cells of a particular type which do not overexpress a given cytokine regulatory factor, *e.g.*, PKR, such as an untransfected cell line which either normally produces or is capable of producing a given cytokine. It will be appreciated that such "normal" cytokine activity or expression, is reported as a range of cytokine activity or expression that is generally observed for a given type of cells which do not overexpress the cytokine regulatory factor and may vary somewhat dependent upon culture conditions.

For example, a given cell line which does not overexpress PKR may have a normal range of cytokine activity which differs from the range of cytokine activity for that same cell line following transfection with, and over-expression of, PKR.

II. Methods of the Invention

The present invention is based on the discovery that the level of cytokine production in mammalian cell culture can be increased by control of the expression or activity of certain proteins that normally regulate cytokine expression *in vivo*.

These factors include cytokine-specific regulatory factors, for example interferon regulatory factors (IRF-1, IRF-3 and IRF-7), cytokine receptors, nuclear factor κB (NF-κB), activator protein-1 (AP-1), nuclear factor IL-6 (NF-IL6), protein kinase C, p38 MAPK, STAT/Jak kinase system factors, and in particular PKR.

Enhancing the expression or activity of any of these regulatory factors will result in a higher than normal level of expression of the genes which encode one or more cytokines. Such

enhanced cytokine expression will result in more efficient and lower cost production of cytokines.

PKR is used herein as an example of a protein capable of regulating cytokine expression, however it will be understood that other cytokine regulatory factors may be used in place of PKR, *e.g.*, PMA, protein kinase C (PKC) inducers, interferon-γ, interferon-α, interferon-β, TNF-α, GM-CSF, EGF and PDGF.

By increasing the expression/activity of a cytokine regulatory factor, *e.g.*, PKR in mammalian cells, cytokine production can be increased. Mammalian cell cultures which express a higher constitutive level of the cytokine regulatory factor, or in which cytokine regulatory factor expression can be induced to higher levels are therefore useful for the production of cytokines.

The method relies on the use cells that overexpress a protein capable of regulating cytokine expression (a cytokine regulatory factor), *e.g.*, including, but not limited to PKR, as the source of cytokines, with no particular method of cytokine regulatory factor over-expression required except that typically a non-microbial inducer is used. However in some cases, viral induction, e.g., together with sodium butyrate treatment is used.

In particular, the method comprises (a) culturing mammalian cells capable of over-expression of the cytokine regulatory factor or an analog or homologue thereof under conditions sufficient to overexpress the cytokine regulatory factor, and (b) treating the cell culture as appropriate to induce the expression of a cytokine gene.

The cells used to produce a given cytokine can overexpress a cytokine regulatory factor, *e.g.*, PKR from any mammalian source, such as the PKR normally found in rabbit reticulocytes, various mouse tissues, or human peripheral blood mononuclear cells. Preferably murine p65 kinase (Feng, G.S *et al.*,1992) and most preferably human p68 kinase (Meurs, E *et al.*, 1990; GenBank Accession No. NM_002759), is overexpressed, in a corresponding murine or human cell culture, respectively.

In some cases, the cytokine regulatory factor which is overexpressed is an analog of a native cytokine regulatory factor, *e.g.*, a PKR analog such as a non-natural protein kinase that can mediate dsRNA activation of cytokine transcription (usually obtained by modification of the gene encoding a native PKR protein).

Mammalian cells capable of overexpressing a cytokine regulatory factor may be obtained by any of a number of methods, that are well known in the art or may be obtained from commercial sources.

Exemplary methods for obtaining cytokine regulatory factor-overexpressing cells include selection for cells that express higher levels of the cytokine regulatory factor, transfection with an expression vector encoding the cytokine regulatory factor under control of a promoter, and other methods which result in an increase in expression of the cytokine regulatory factor over normal levels.

Additional approaches include inactivation or decreasing the levels of the PKR-inhibiting factor, p58 which normally inhibits PKR activity. Mutation, modification or gene-targeting ablation of p58 will result in enhanced PKR activity (Barber, G.N. *et al.*, 1994).

Another example includes natural, synthetic or recombinant activators of PKR that can enhance the expression of PKR, *e.g.* the PKR activator protein, PACT (Patel, R.C. and Sen, G.C., 1998).

The present invention provides vectors suitable for the transformation of human cells, *e.g.*, recombinant expression vectors containing a polynucleotide which encodes a protein effective to regulate cytokine expression operably linked to regulatory elements effective for expression of the protein in a mammalian cell line. Preferred coding sequences include the coding sequence for p68 (human) or p65 (murine) kinase. In a related aspect, the invention includes a mammalian host cell containing the vector.

In one preferred approach, the vector includes a polynucleotide sequence which encodes a protein effective to regulate cytokine expression together with additional coding sequences, such as fusion protein or signal peptide coding sequences, in combination with non-coding sequences, such as introns and control elements, such as promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in a suitable host, and/or in a vector or host environment in which the coding sequence is a heterologous gene. By "heterologous DNA" and "heterologous gene" is meant nucleotides that are not endogenous to the cell or part of the genome in which they are present. In general, such nucleotides have been added to the cell, by transfection, homologous recombination, microinjection, electroporation, or the like. The expression vector also contains a ribosome binding site for translation initiation, and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells.

Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. Appropriate cloning and expression vectors for use in human cells are also described in Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Press, Plainview, N.Y. and Ausubel FM *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., expressly incorporated by reference herein. Exemplary promoters include both constitutive promoters and inducible promoters, examples of which include a CMV promoter, an SV40 early promoter, an RSV promoter, an EF-1α promoter, a promoter containing the tet responsive element (TRE) and the metallothienein promoter.

A heterologous nucleic acid sequence which contains the coding sequence for a protein effective to regulate cytokine expression may be included in any one of a variety of expression vectors for expressing a polypeptide. Any vector may be used as long as it is replicable and viable in the mammalian cells into which it is introduced. The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by standard procedures. Such procedures and related sub-cloning procedures are deemed to be within the scope of knowledge of those skilled in the art.

The vector containing the appropriate DNA sequence as described above, as well as an appropriate promoter or control sequence, may be employed to transform a mammalian cell line to permit the cells to express the protein and thereby enhance cytokine production.

Introduction of the vector into a host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofectamine or lipofection-mediated transfection, or electroporation (Davis, L., Dibner, M., and Battey, I. Basic Methods in Molecular Biology, 1986). For long-term, high-yield production of recombinant cytokines, stable expression is preferred. It follows that any method effective to generate stable transformants may be used in practicing the present invention.

The invention also provides host cells which have been transduced, transformed or transfected with an expression vector of the invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to those skilled in the art.

Examples of appropriate clonal cell lines for expression of a cytokine-regulating factor include, but are not limited to Namalwa, U937, Vero, MRC-5, WI-38 cells, Flow 1000 cells, Flow 4000 cells, FS-4 and FS-7 cells, MG-63 cells, CCRF-SB cells , CCRF-CEM cells and T98G cells. Examples of appropriate primary cell types for expression of a cytokine-regulating factor include, but are not limited to cells of the monocyte/macrophage lineage, lymphocytic lineage cells including T- and B-cells, mast cells, fibroblasts, bone marrow cells, keratinocytes, osteoblast derived cells, melanocytes, endothelial cells, platelets, various other immune system cells, lung epithelial cells, pancreatic parenchmal cells, glial cells and tumor cells derived from such cell types.

Preferably the cytokine-regulating factor-overexpressing cell culture will be inducible for over-expression of the cytokine-regulating factor in order to regulate the level of the factor available for cytokine induction.

By over-expression of a cytokine-regulating factor is meant higher than normal levels of cytokine-regulating factor activity. "Normal" cytokine-regulating factor activity or expression is reported as a range of cytokine-regulating factor activity or expression, which is generally observed for a given type of cells which have not been transfected with a vector encoding the cytokine-regulating factor, are unstimulated (not induced or primed) and uninfected. It will be understood that the range of normal cytokine-regulating factor activity will vary dependent upon the particular factor, cell type and for a given cell type may vary somewhat dependent upon culture conditions.

Higher than normal level preferably means at least 150%, more preferably at least 200 or 300%, most preferably at least 500%, of the normal level for a given cytokine-regulating factor under the particular culture conditions employed. The cytokine-regulating factor-overexpressing cell culture may be constitutive for over-expression of the cytokine-regulating factor or inducible for over-expression of the cytokine-regulating factor.

In one preferred embodiment, the cytokine-regulating factor is PKR and the PKR-overexpressing cell culture is inducible for PKR over-expression in order to regulate the level

of PKR available for cytokine induction. Any of a number of known cell types are useful for making a PKR-overexpressing cell line, with particular examples provided above.

The activity of a given cytokine-regulating factor can be determined by methods known in the art. By way of example, assays for PKR expression include autophosphorylation assays, assay for eIF2α phosphorylation, Western blot analysis for protein levels and Northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) for PKR mRNA.

In general, additional steps are taken to enhance expression of cytokines by mammalian cells. Such steps include one or more of (1) culturing the transfected cells under conditions effective to enhance expression of the cytokine-regulating factor, (2) priming the cytokine-regulating factor-expressing cells with reagents including but not limited to polypeptides, chemicals, or nucleic acids, and (3) treating the cytokine-regulating factor-expressing cells to induce cytokine production (induction).

Priming may include treating with a priming agent, *e.g.*, phorbol myristate acetate (PMA) and other phorbol esters, calcium ionophores, interferon-α, interferon-γ, interferon-β, G-CSF, GM-CSF, PDGF, TGF, EGF, sodium butyrate, a kinase activator or a transcription activator.

Treating may include adding a microbial (i.e., viral) or non-microbial inducer to the cell culture. In general, the inducer will be a non-microbial inducer, *e.g.*, poly(I):poly(C) or poly r(I):poly r(C).

In general, U937 cells are preferred for production of FGF and sTNF-R; Jurkat cells are preferred for production of IL-3 and TNF-β; fibroblasts are preferred for production of FGF and angiostatin; U937 cells are preferred for production of IL-6 and homologs thereof; CD-4 expressing cells including Jurkat and HUT are preferred for production of TNF-β; and T and B-cells including Jurkat and Namalwa are preferred for production of IL-8 and homologs thereof.

Once increased expression of a given cytokine is achieved, the cytokine thereby produced is purified from the cell culture. Exemplary procedures suitable for such purification include the following: antibody-affinity column chromatography, ion exchange chromatography; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, METHODS IN ENZYMOLOGY, 182, 1990; Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Springer-Verlag, New York, 1982. The purification step(s) selected will depend, for example, on the nature of the production process used and the particular cytokine produced.

In one preferred aspect of the invention, a combination of culture conditions, priming and treating results in significantly enhanced cytokine production, *e.g.*, an increase of at least 2.5-fold, and preferably an increase of 10 fold or greater. In some cases, the methods of the invention result in an increase in cytokine production that is 100 to 1000 fold or more.

Problems typically associated with production of cytokines in cell culture, for example low yield from non-recombinant mammalian systems, improper glycosylation, or misfolding of

proteins produced in microbial systems are eliminated in the methods of the present invention, which should prove to be useful for the development of therapeutic proteins.

III. Cytokines

Cytokines elicit their biological activities by binding to their cognate receptors followed by signal transduction leading to stimulation of various biochemical processes. In some cases, the expression of such receptors is regulated by specific signals, for example a cytokine may be involved in positive or negative feedback loops and thereby regulate the expression of the receptor for the same or a different cytokine. Such receptors may be the same type of cell that produces the cytokine or a different type of cell.

Cytokines serve to mediate and regulate immune and inflammatory responses. In general, cytokine production is transient and production takes place during a short period of transcription resulting in production of mRNA transcripts which are also short-lived and subject to post-transcriptional control mechanisms. Recent studies have indicated that a common signal transduction pathway, the "Jak/STAT" pathway, is used by a variety of cytokines (Abbas, AK, *et al.*, 1997).

It will be appreciated that the cellular source of cytokines is a distinguishing characteristic of each individual cytokine capable of production by multiple diverse types of cells. In addition, a given cytokine (1) may act on more than one type of cells, (2) may have more than one effect on the same cell, (3) may have an activity shared with another cytokine, and (4) may influence the synthesis or effect of other cytokines, *e.g.*, by antagonizing, or synergizing the effects thereof.

Recombinant soluble-tumor necrosis factor receptor (sTNF-R) has been observed to neutralize TNF and to have anti-inflammatory properties.

sTNF-R is currently produced as a recombinant protein.

Target cells for production of sTNF-R include cells of the monocyte/macrophage lineage.

Clinical utilities for sTNF-R include applications to rheumatoid arthritis and sepsis as well as various research applications.

Interleukin-2 (IL-2), or "T-cell growth factor" is known to promote T-cell growth and function. When T helper lymphocytes have been activated by stimulation with certain mitogens or interaction of the T cell receptor complex with antigen/MHC complexes on the surface of antigen-presenting cells, IL-2 and IL-2 receptors are induced resulting in clonal expansion of antigen-specific T cells. (See, *e.g.*,. Smith, KA, 1988; Dinarello, 1994.).

IL-2 is currently produced by the expression of recombinant IL-2 protein in various types of cells.

Target cells for production of IL-2 include T-cells.

Clinical utilities for IL-2 include anti-cancer and anti-HIV applications, as well as various research uses.

Interleukin-3 (IL-3) has been implicated as a stimulatory factor in the formation of a number of types of hematopoietic cells, including granulocytes, macrophages, eosinophils, mast cells, megakaryocytes and erythroid cells.

IL-3 has been observed to have multiple hematopoietic effects on bone marrow (BM) stem cells, which are more pleiotropic than G-CSF.

IL-3 is currently produced using recombinant DNA technology.

Target cells for production of IL-3 include T-cells and mast cells.

Clinical utilities for IL-3 include applications to: (1) T- and stem cell expansion *ex vivo*, (2) post-BM transplant *in vivo*, (3) bone marrow failure (chemotherapy), (4) blood dyscrasia and (5) pancytopenia, in addition to various research uses.

Interleukin-4 (IL-4), also known as B cell stimulating factor, or BSF-1 has been observed to have numerous biological effects including, (1) co-stimulation of T cells, mast cells, granulocytes, megakaryocytes, and erythrocytes, (2) induction of the expression of class II major histocompatibility complex molecules on resting B cells, (3) enhanced secretion of IgE and IgG1 isotypes by stimulated B cells, and (4) anti-inflammatory properties.

IL-4 has been observed to have effects on type 2 helper T-cell (TH2) responses and anti-growth properties.

IL-4 is currently produced by expression as a recombinant protein.

Target cells for production of IL-4 include T-cells and mast cells.

Clinical utilities for IL-4 include applications to stem cell expansion *ex vivo*, anti-cancer strategies and treatment of solid tumors, in addition to various research uses.

Interleukin-5 (IL-5), is proposed to have effects on suppression of allergic responses, and suppression of eosinophils.

IL-5 is currently produced by expression as a recombinant protein.

Target cells for production of IL-5 include T-cells and mast cells.

Clinical utilities for IL-5 include applications in treatment of asthma, in addition to various research uses.

Interleukin-6 (IL-6), is a multi-functional cytokine produced by various types of cells, and acts as a differentiation and growth factor on various types of cells including cells in the immune system, hepatocytes, kidney cells, hematopoietic staminal cells, keratinocytes and neurons.

IL-6 has been observed to play a role in inflammation, and have to anti-growth properties.

IL-6 is currently produced by expression as a recombinant protein.

Target cells for production of IL-6 include fibroblasts, T-cells and cells of the monocyte/macrophage lineage.

Clinical utilities for IL-6 include applications to the treatment of breast cancer and leukemia, and the anti-inflammatory properties of IL-6 may be useful in the treatment of various infectious diseases and thrombopoietic disorders, in addition to various research uses.

Interleukin-7 (IL-7), previously known as "lymphopoietin-1", was originally defined by its ability to stimulate proliferation of pre-B cells (B220+ cells) derived from long-term bone

marrow culture (Whitlock *et al.*, 1984). IL-7 has been observed to stimulate the growth of B- and T-cell progenitors in bone marrow, and to play a role in (1) cytokine synthesis in the skin, (2) increased cytotoxic T lymphocyte (CTL) activity, and (3) increased natural killer (NK) cell activity.

IL-7 is currently produced by expression as a recombinant protein.

Target cells for production of IL-7 include bone marrow cells and keratinocytes.

Clinical utilities for IL-7 include applications to the treatment of melanoma, in addition to various research uses.

Interleukin-8 (IL-8), is a chemotactic cytokine, capable of causing degranulation of human neutrophils and produced by keratinocytes, epithelial cells, synoviocytes, hepatocytes, monocytes and neutrophils. The biological effects of IL-8 have been described as mediated through seven transmembrane domain, G-protein-coupled receptors.

IL-8 has been observed to be a member of the CXC chemokine family, and to have chemotactic and angiogenic properties.

IL-8 is currently produced by expression as a recombinant protein.

Target cells for production of IL-8 include fibroblasts, T-cells and cells of the monocyte/macrophage lineage.

Although clinical studies have not been completed, predicted utilities for IL-8 include applications to the treatment of bacterial and viral infections, in cancer therapy and in promoting vessel growth, in addition to various research uses.

Tumor necrosis factor-alpha (TNF-α) and tumor necrosis factor beta (TNF-β; lymphotoxin), are produced primarily by macrophages and lymphocytes, respectively. TNF-α has numerous biological functions, including hemorrhagic necrosis of transplanted tumors, cytotoxicity, an important role in endotoxic shock and in inflammatory, immunoregulatory, proliferative, and antiviral responses. TNF-alpha and TNF-beta share a similar spectrum of biological activities. TNF-β shows anticellular activity on neoplastic cell lines but not on primary cell cultures and normal cell lines, suggesting that it has potent anti-tumor activity. TNF-beta also plays an important role in lymphoid organ development. TNF-α and TNF-β bind to the same cell surface receptors consistent with their similar activities.

Clinical utilities of TNF-α and TNF-β include anti-cancer applications particularly in combination with other chemotherapeutic agents or with gene therapy. Combination therapy may be important so that a relatively low dose of TNF can be administered (U.S. Patent No. 5,976,800 issued 11/2/99). The agents may be useful for treatment of patients with disseminated tumors including melanoma, osteosarcoma, breast carcinoma, and lymphomas.

Granulocyte colony-stimulating factor (G-CSF), has been observed to stimulate the proliferation and differentiation of neutrophil precursors and to play a role in granulocyte maturation and oxidative bursts.

G-CSF is currently produced by expression as a recombinant protein in bacterial and mammalian host cells.

Target cells for the production of G-CSF include fibroblasts, endothelial cells and cells of the monocyte/macrophage lineage.

Clinical utilities for G-CSF include application to the treatment of post-chemotherapy pancytopenia, treatment following bone marrow transplantation, and various research uses.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been observed to stimulate the production of colonies of granulocytes and macrophages from their precursor cells and to promote the growth and differentiation of pluripotent progenitor cells. In addition, GM-CSF appears to play an effector role in granulocyte and macrophage functions.

GM-CSF is currently produced by expression of recombinant protein in bacterial and mammalian host cells.

Target cells for production of GM-CSF include fibroblasts, endothelial cells and T-cells.

Clinical utilities for GM-CSF include broad application to the treatment of post-chemotherapy pancytopenia, treatment following bone marrow transplantation, and various research uses.

Fibroblast growth factor (FGF), both acidic & basic forms, have been observed to play a role in angiogenesis and endothelial cell proliferation. Studies with recombinant FGF-5 in culture indicate that it can promote the survival of cultured motor neurons, indicating that FGF-5 is a neurotrophic factor of motor neurons.

FGF is currently produced by expression as a recombinant protein.

Target cells for production of FGF include platelets, endothelial cells and cells of the macrophage lineage.

Utilities for FGF include application to the treatment of ischemic heart disease, congestive heart failure, and various condition involving the inflammatory response, as well as to various research uses.

Vascular Endothelial Growth Factor (VEGF), is an angiogenic growth factor with numerous physiologic effects, including angiogenesis, nitric oxide-mediated vasodilation, increased vascular permeability, and increased endothelial cell proliferation and motility. Expression of VEGF has been observed to be significantly upregulated by ischemia.

VEGF is currently produced by expression of the recombinant protein in microbial and mammalian host cells.

Target cells for production of VEGF include various immune cells.

Utilities for VEGF include applications to the treatment of congestive heart failure, in addition to various research uses.

Platelet-derived growth factor (PDGF-1 and PDGF-2), have been observed to act as growth factors and to play a role in angiogensis. Purified human platelet-derived growth factor (PDGF) has been shown to be a mitogen for mesenchymal-derived cultured smooth muscle cells, fibroblasts and glial cells and to act as a chemoattractant for monocytes and neutrophils.

In vivo, PDGF is found in the alpha granules of platelets and in endothelial cells, and has been shown to have wound-healing properties.

PDGF-1 and PDGF-2 are currently produced by expression as a recombinant protein.

Target cells for production of PDGF-1 and PDGF-2 include platelets, endothelial cells and cells of the monocyte/macrophage lineage.

Utilities for PDGF-1 and PDGF-2 include applications to the treatment of ischemic heart disease, and various research uses.

Angiostatin and endostatin have been observed to play a role in the inhibition of endothelial cell growth. Angiostatin and endostatin have been observed to act as inhibitors of angiogenesis in tumor bearing animals (Lannutti BJ *et al.*, 1997; O'Reilly MS, *et al.*, 1997).

Target cells for production of angiostatin and endostatin include various immune system cells.

Utilities for angiostatin and endostatin include applications in the treatment of cancer, and various research uses.

Additional Cytokines

Other exemplary cytokines the expression of which may be increased using the methods of the invention include, but are not limited to additional family members of the above mentioned cytokines, such as the IL-6 family members oncostatin M; IL-11, leukemia inhibitory factor (LIF); ciliary neurotrophic factor and cardiotrophin. In addition, the expression of cognate receptors for the above mentioned cytokines may be increased, such as TNF soluble receptor (sTNF-R), Fas soluble receptor (sFas), and the IL-6 family receptor, soluble glycoprotein 130 (gp 130). Further exemplary cytokines the expression of which may be increased using the methods of the invention include, but are not limited to cytokines whose genes are regulated by transcriptional factors activated by PKR, including NF-κB, IRF-1,3 and 7 and Stat family members.

IV. PKR

In one exemplary approach, PKR is the factor used to enhance cytokine production and the cells used in practicing the methods of the invention are capable of producing PKR and will produce a baseline level of PKR in the absence of modification.

In some cases, PKR activity may be evaluated directly, by measuring PKR autophosphorylation activity, or the activity of the kinase in phosphorylating a substrate, preferably eukaryotic initiation factor-2 alpha (eIF-2 alpha), or another factor such as nuclear factor-kappa B (NF-κB) (Link *et al.*, 267 J. Biol. Chem. 239, 1992), or by a biochemical tests such as polymerase chain reaction (PCR), or Western blotting with a PKR specific antibody.

A number of approaches may be taken to enhance PKR expression. In one embodiment of the invention, PKR expression is enhanced by a transfecting a mammalian host cell capable of producing a given cytokine with an expression vector comprising a sequence encoding PKR operably linked to a promoter and containing control sequences necessary for expression of PKR by the mammalian host cell.

Host cells transfected with a nucleotide sequence encoding PKR may be cultured under conditions suitable for the expression of the encoded PKR in the cells. As will be understood by those of skill in the art, expression vectors containing polynucleotides encoding PKR can be designed such that the PKR produced by a recombinant cell may be secreted, membrane-bound, or contained intracellularly depending on the sequence and/or the vector used.

In some cases, additional steps may be taken to enhance PKR expression by transfected host cells. Such steps include one or more of (1) culturing the transfected cells under conditions effective to enhance PKR expression, (2) priming the PKR-transfected cells, and (3) treating the PKR-transfected cells to induce cytokine production as further described below.

In a preferred embodiment a combination of culture conditions, priming and treating results in significantly enhanced cytokine production, *e.g.*, an increase of at least 2.5-fold and preferably an increase of 10 fold or greater. In some cases, the methods of the invention result in an increase in cytokine production that is 100 to 1000 fold or more.

V. Evaluation Of Cytokine Expression

In order to evaluate the expression of a cytokine of interest by a cytokine regulatory factor-overexpressing cell line, assays can be carried out at the protein level, the RNA level or by use of functional bioassays particular to the individual cytokine being expressed.

Immunoassays for a particular cytokine protein may be carried out using procedures routinely employed by those of skill in the art. Such immunoassays can be used to qualitatively and quantitatively analyze expression of a cytokine of interest.

A purified form of the cytokine of interest may be obtained from a natural source or produced recombinantly in transfected cells, and purified using standard techniques for protein purification. The purified protein may then be used to produce either monoclonal or polyclonal antibodies specific to the expressed protein, which can be used in various immunoassays. (See, *e.g.*, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Pubs., N.Y., 1988). Exemplary assays include ELISA, competitive immunoassays, radioimmunoassays, Western blots, indirect immunofluorescent assays and the like.

In general, such antibodies are commercially available. Cytokine analysis kits which are also commercially available are typically used for the quantitative immunoassay of the expression level of known cytokines.

Specific examples of the steps described above are set forth in the following examples. However, it will be apparent to one of ordinary skill in the art that many modifications are possible and that the examples are provided for purposes of illustration only and are not limiting of the invention unless so specified.

EXAMPLE 1

Preparation Of The PKR Overexpressing Namalwa Cell Line.

cDNA encoding the full-length human PKR molecule (551 amino acids; Meurs, E *et al.*, 1990; GenBank Accession No. NM_002759) was inserted into a eukaryotic expression vector, such that the PKR coding sequence was expressed under the control of the CMV promoter. The vector contains various features suitable for PKR transcription, including: i) promoter sequences from the immediate early gene of the human CMV (cytomegalovirus) for high level mRNA expression; ii) polyadenylation signal and transcription termination sequences from the β-globin gene to enhance RNA stability; iii) the ampicillin resistance gene;

and iv) the ColE1 origin of replication for selection and maintenance in E. coli. A second vector contained the ampicillin resistance gene and the ColE1 origin for selection and maintenance in E. coli. and the G418 resistance marker (Neo) to allow for selection and identification of the plasmids after co-transfection into eukaryotic cells.

Stable transfectants were obtained by electroporation of $4x10^6$ exponentially growing Namalwa cells with 15 μg of the PKR expressing plasmid and 15 μg of the Neo containing vector in DMEM/F12 (+10% FBS) using a Gene Pulser apparatus (BioRad) set at 800μF, 300V. Bulk populations of stable transfectants were obtained by selection with 2 mg/ml geneticin (Gibco-BRL) for 3-4 weeks. Clonal lines were subsequently obtained by limiting dilution cloning. The level of PKR in the parental and PKR- transfected Namalwa cells was analyzed and found to have increased approximately sixteen-fold in the PKR-transfectants relative the parental Namalwa cells.

One representative PKR-overexpressing and clonal Namalwa cell line was selected and designated 2A1.D1.G7.

2A1.D1.G7 and parental Namalwa cells were cultured at $2.5x10^5$ cells/ml in DMEM/F12 medium supplemented with 10% FBS. The cells were treated with 20 nM PMA (priming) for 20 hr followed by treatment by 200 μg/ml poly r(I):r(C) (induction) for 3 days. One set of cells was left untreated (non-induced controls). Following treatment, the culture supernatants were collected and analyzed for interleukin-6 (IL-6), interleukin-8 (IL-8), and TNF-β levels by ELISA according to the procedure provided by the supplier of the ELISA kits (R&D Systems).

The production of IL-6 under various conditions is shown in Figure 1. Neither the parental Namalwa nor 2A1.D1.G7 cell lines produced IL-6 in the absence of the priming agent (PMA) and inducing agent [poly r(I):r(C)]. The parental Namalwa cells treated with PMA and poly r(I):r(C) also did not produce detectable IL-6 (in an assay with a minimum detectable level of 3 pg/ml). In contrast, 2A1.D1.G7 treated with PMA and poly r(I):r(C) produced greater than 300 pg/ml of IL-6. This represents at least a 100-fold increase in IL-6 production over untreated 2A1.D1.G7 cells and PMA and poly r(I):r(C) treated parental Namalwa cells. In addition, several other PKR-overexpressing clonal cell lines produced greater than 300 pg/ml of IL-6 following PMA and poly r(I):r(C) treatment (data not shown). These results indicate that overexpressing the PKR gene in Namalwa cells and subsequent priming and activation results in the overexpression of IL-6.

The production of IL-8 under various conditions is shown in Figure 2. Consistent with the results obtained for IL-6, neither the parental Namalwa nor 2A1.D1.G7 produced IL-8 in the absence of the priming agent (PMA) and inducing agent [poly r(I):r(C)]. The parental Namalwa cells treated with PMA and poly r(I):r(C) also did not produce detectable IL-8 (in an assay with a minimum detectable level of 31 pg/ml). In contrast, 2A1.D1.G7 cells treated with PMA and poly r(I):r(C) yielded approximately 300 pg/ml of IL-8, which represents at least a 10-fold increase over the untreated cells and the PMA and poly r(I):r(C) treated parental Namalwa cells. In addition, several other PKR-overexpressing clonal cell lines produced between 250-470 pg/ml of IL-8 following PMA and poly r(I):r(C) treatment (data not shown).

The production of TNF-β under various conditions is shown in Figure 3. In this case, neither parental Namalwa nor 2A1.D1.G7 produced TNF-β in the absence of priming and induction. However, the parental Namalwa cell line produced approximately 800 pg/ml of TNF-β following priming and induction, while the 2A1.D1.G7 cell line produced greater than 2000 pg/ml. Again, the PKR-overexpressing cell line produced more cytokine than the parental cell line (approximately a 2.5-fold increase) following PMA and poly r(I):r(C) treatment. In addition, several other PKR-overexpressing clonal cell lines produced greater than 2000 pg/ml of TNF-β following PMA and poly r(I):r(C) treatment (data not shown).

The results presented herein show that the present invention provides an effective method for enhancing the production of cytokines in mammalian cell culture.

IT IS CLAIMED:

1. A method for producing a cytokine in mammalian cell culture, comprising:
(a) culturing a mammalian cell line capable of producing the cytokine;
(b) modifying said cultured mammalian cell line in a manner effective to result in over-expression of a cytokine regulatory factor in the cell line, wherein above-normal levels of the factor capable of regulating cytokine expression are obtained in said cell line;
(b) treating said cultured, cytokine regulatory factor-overexpressing mammalian cell line to effect cytokine production; and
(c) collecting said cytokine produced by the cultured, treated cell line.

2. The method of claim 1 wherein said cytokine regulatory factor is PKR.

3. The method of claim 1 wherein said modifying means transfecting said cultured mammalian cell line with an expression vector containing DNA encoding the cytokine regulatory factor operably linked to a promoter, under conditions that result in overexpression of the cytokine regulatory factor in said transfected cells.

4. The method of claim 1, wherein said treating comprises a priming step.

5. The method of claim 4, wherein said treating further comprises an inducing step.

6. The method of claim 4, wherein said priming is accomplished by treatment with a phorbol ester.

7. The method of claim 5, wherein said inducing is accomplished by treatment with poly r(I):poly r(C).

8. The method of claim 2, wherein said cytokine is selected from the group consisting of interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor beta (TNF-β).

9. The method of claim 1, wherein said promoter is an inducible promoter.

10. The method of claim 9, wherein said inducible promoter is a metallothionein promoter or a tetracycline (TRE) promoter.

11. The method of claim 8, wherein said promoter is a CMV promoter.

12. The method of claim 8, wherein said cytokine is interleukin 6 (IL-6) and the mammalian cell line is Namalwa.

13. The method of claim 8, wherein said cytokine is interleukin 8 (IL-8) and the mammalian cell line is Namalwa.

14. The method of claim 8, wherein said cytokine is tumor necrosis factor beta (TNF-β) and the mammalian cell line is Namalwa.

15. A mammalian cell line composition characterized by above normal expression of PKR and above normal expression of interleukin 6 (IL-6).

16. A mammalian cell line composition characterized by above normal expression of PKR and above normal expression of interleukin 8 (IL-8).

17. A mammalian cell line composition characterized by above normal expression of PKR and above normal expression of tumor necrosis factor beta (TNF-β).

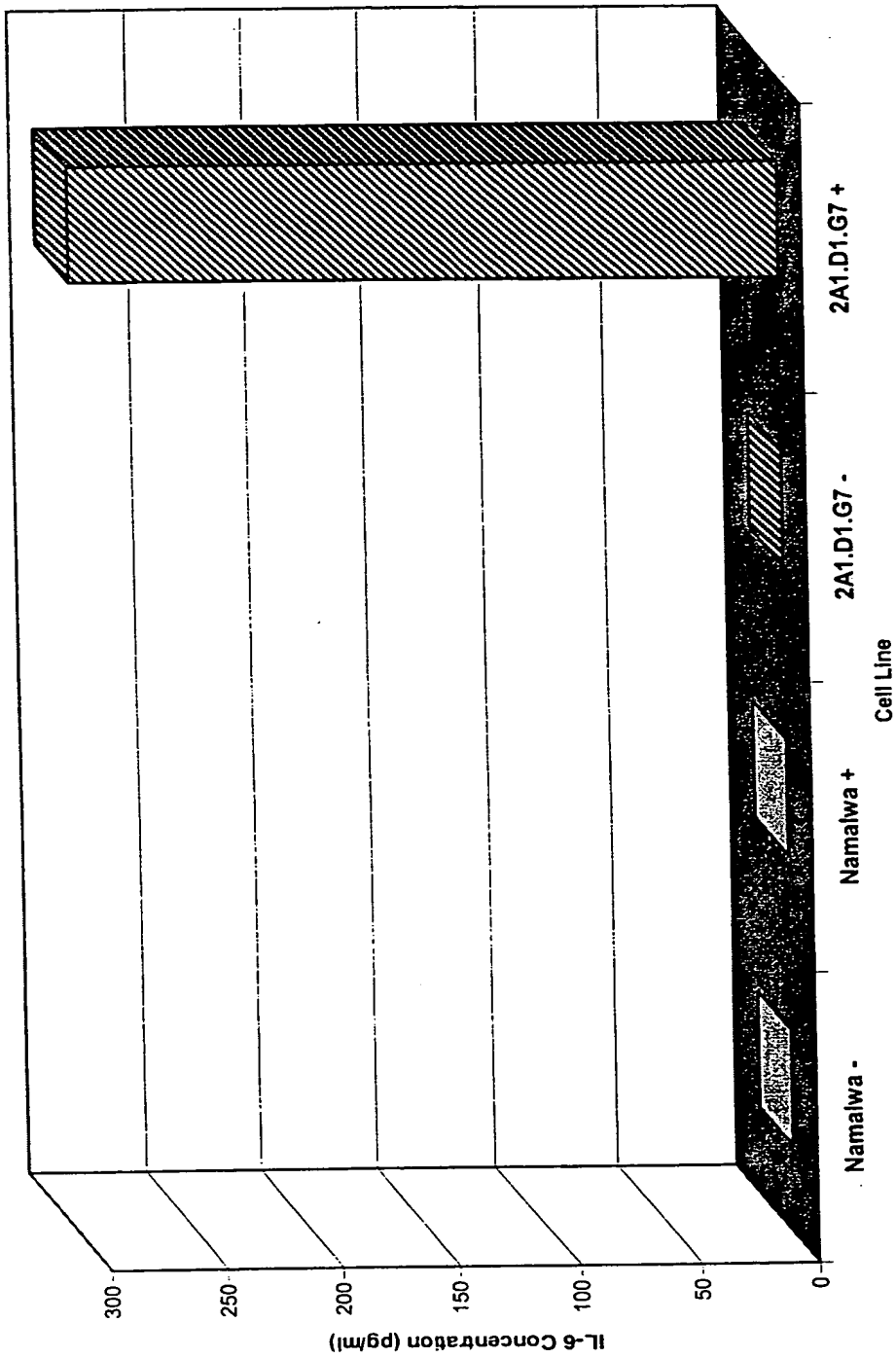
IL-6 Concentration (pg/ml)
300
250
200
150
100
50
0
Namalwa -
Namalwa +
2A1.D1.G7 -
2A1.D1.G7 +
Cell Line

Fig. 1

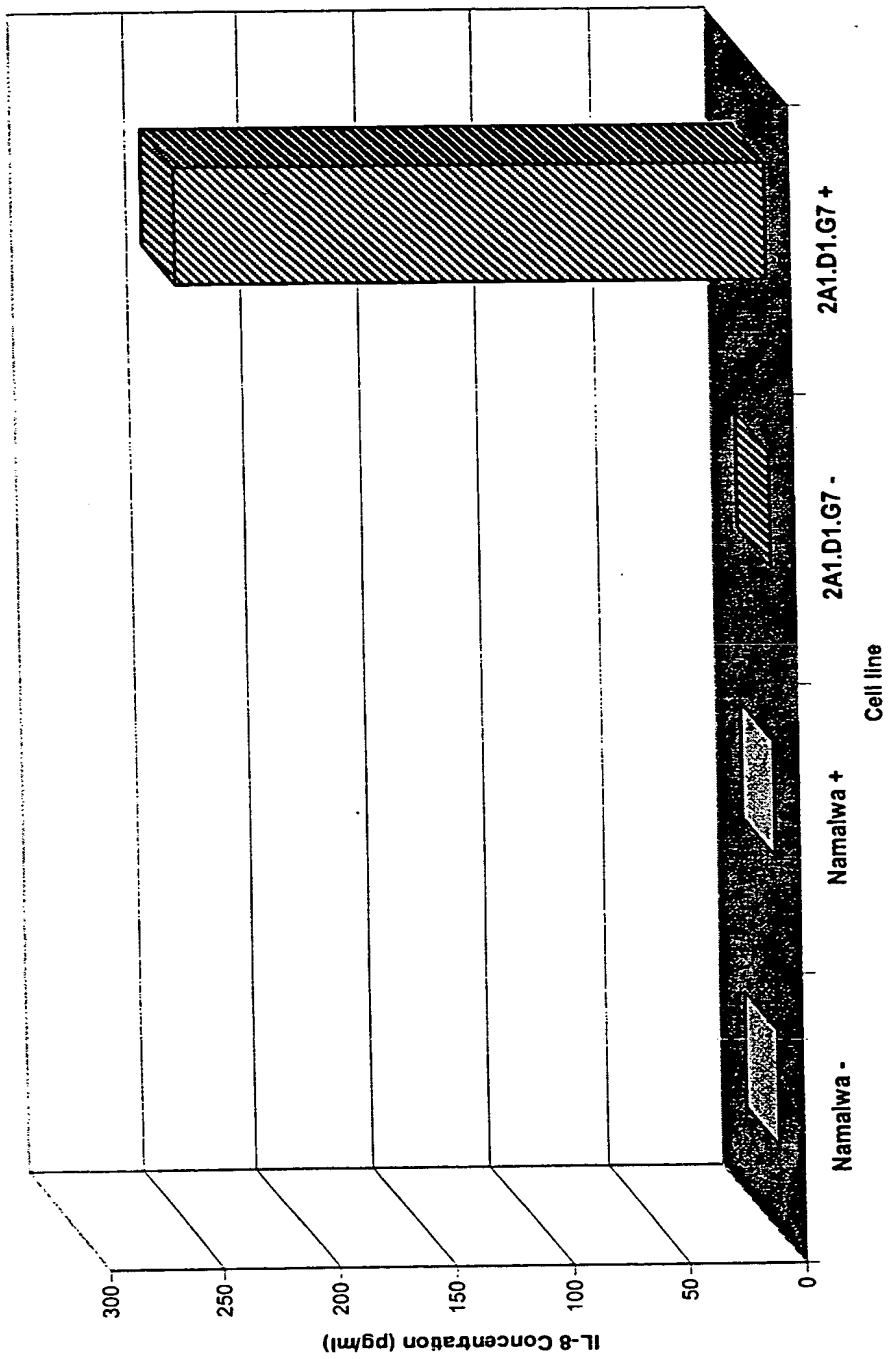
IL-8 Concentration (pg/ml)
300
250
200
150
100
50
0
Namalwa -
Namalwa +
2A1.D1.G7 -
2A1.D1.G7 +
Cell line

Fig. 2

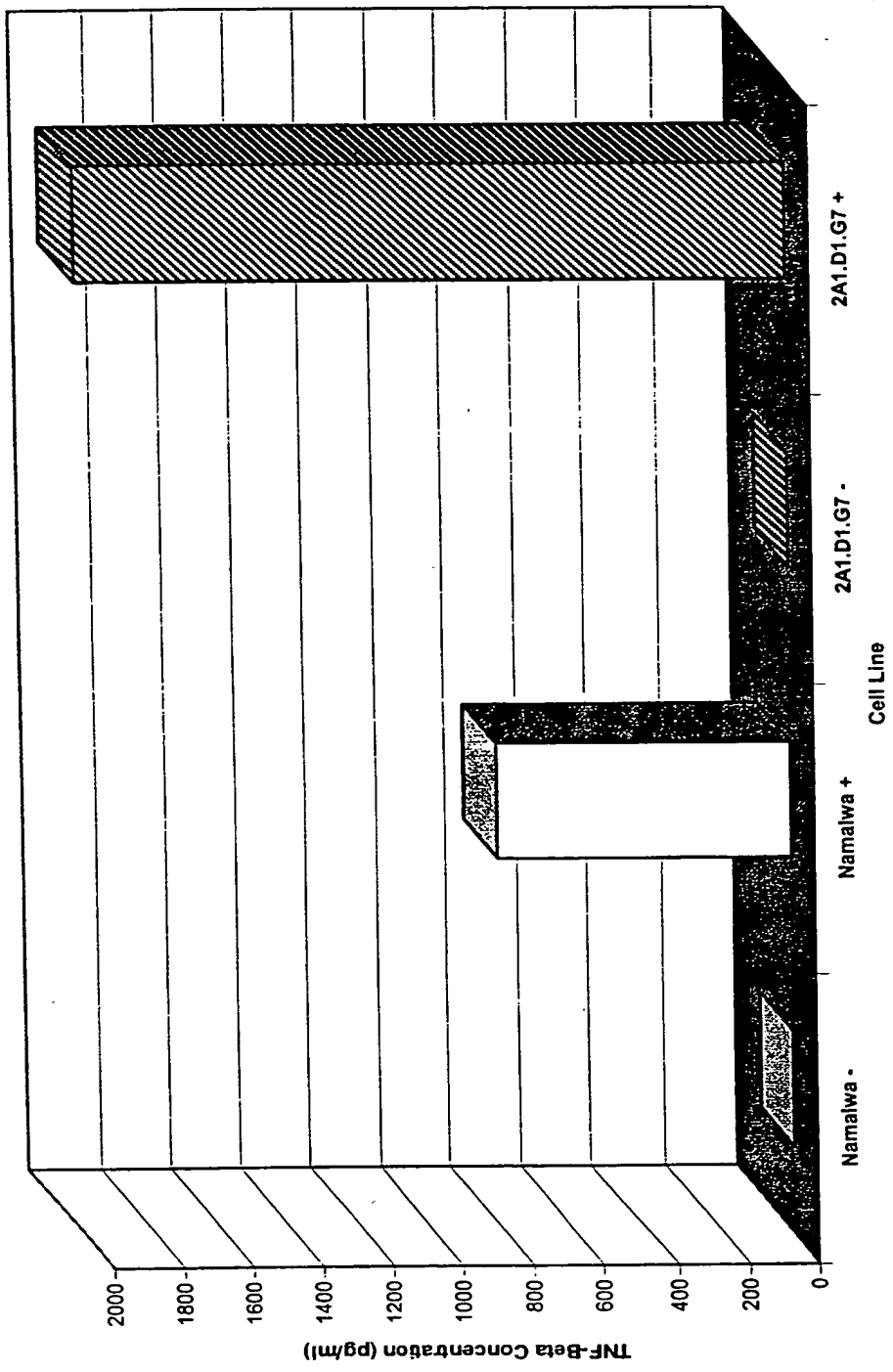
TNF-Beta Concentration (pg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Namalwa -
Namalwa +
2A1.D1.G7 -
2A1.D1.G7 +
Cell Line

Fig. 3